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# Amphiphilic and hydrophilic forms of acetylcholinesterase from sheep platelets

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**Abstract**

Acetylcholinesterase (AChE, EC 3.1.1.7) was extracted from sheep platelets by successive homogenizations, yielding low-salt soluble (LSS), high-salt soluble (HSS) and detergent-soluble (DS) fractions. These accounted, respectively, for about 30%, 7% and 60% of total AChE activity. Applications of hydrophobic chromatography on phenyl-agarose to three solubilized fractions revealed that hydrophilic forms were almost exclusively located in the LSS fraction (≈27% of total AChE), whereas most amphiphilic forms were present in DS extracts (≈59% of total AChE), the remaining forms being distributed among aqueous soluble fractions. Enzyme molecular forms in the solubilized extracts were identified by centrifugation in 5–20% sucrose gradients containing Triton X-100 or Brij 97 to differentiate between hydrophilic or amphiphilic species. A predominance of hydrophilic dimeric forms (≈22%), with small amounts of hydrophilic monomers (5%) and amphiphilic dimers and monomers (3%), was found in soluble AChE (LSS fraction). Amphiphilic AChE forms extracted in the HSS and DS fractions had a single peak in the sedimentation profiles with sedimentation coefficients of about 6S in gradients with Triton X-100; these were slightly shifted in the presence of Brij 97. After treatment with dithiothreitol, this molecular form solubilized in DS was converted to another molecular form with a lower sedimentation coefficient. Our results show that amphiphilic globular dimers are the dominant molecular form in sheep platelet AChE, suggesting a partial conversion of this membrane-bound form into soluble dimers and monomers, mainly with a hydrophilic character, through the action of either endogenous proteases and phospholipases or residual endogenous reducing agents. 

*Keywords:* Acetylcholinesterase; Amphiphilic form; Hydrophilic form; Molecular form; Platelet; (Sheep)

## 1. Introduction

Acetylcholinesterase (AChE, EC 3.1.1.7) is a ubiquitously distributed glycoprotein that is associated primarily with cells involved in cholinergic synaptic transmission, where it hydrolyzes the neurotransmitter acetylcholine [1,2]. Nevertheless, it is also found in other vertebrate tissues [3] and blood cells, such as erythrocytes [4], lymphocytes [5,6] and platelets [7,8]. The function of blood-borne AChE is uncertain. It

Abbreviations: AChE, acetylcholinesterase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DS, detergent-soluble; DTT, dithiothreitol; $G_2^A$, amphiphilic dimer; $G_1^A$, amphiphilic monomer; $G_2^H$, hydrophilic dimer; $G_1^H$, hydrophilic monomer; GPI, glycosylphosphatidylinositol; HSS, high-salt soluble; LSS, low-salt soluble; NEM, *N*-ethylmaleimide; OG, β-D-octylglucoside; PI-PLC, phosphatidylinositol-specific phospholipase C

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seems unlikely that the enzyme would play a hydrolytic effect on circulating acetylcholine, suggesting that it might participate in the maturation and/or degradation of neuropeptides and xenobiotics as well as in pain relief [1,9], because it has been shown to possess other catalytic activities in addition to esterase activity [9].

On the basis of its quaternary structure, AChE is generally classified into two groups: (1) asymmetric species (A), which contain three ($A_{12}$), two ($A_8$) or one ($A_4$) tetrameric assemblies of enzyme subunits disulfide-bonded to a single strand of triple helical collagenic structural subunit; (2) globular forms (G), which have four ($G_4$), two ($G_2$) or one ($G_1$) catalytic subunits. The size and shape of these different molecular forms is reflected in their sedimentation coefficients following sucrose density gradient centrifugation [1,2]. Globular forms are more widely distributed and may exist as either soluble or membrane-bound forms; they can be extracted from tissues with low or high ionic strength buffers without or with detergents. AChE forms can be further subclassified as amphiphilic molecules ($G^A$), in which case they interact with detergents or with hydrophobic resins and presumably correspond to integral membrane proteins in situ, and as hydrophilic components ($G^H$), when they do not and are presumably soluble proteins. In both cases, the forms show similar catalytic properties [1,2,10].

The polymorphism of AChE in blood cells may be limited to globular forms, among which the amphiphilic dimer seems to be the dominant molecular form and has been identified in mammalian erythrocytes [4,11] and human and rabbit lymphocytes [5,6]. It is externally anchored to the membrane by glycosylphosphatidylinositol (GPI) [4,6]. In platelets, most of the AChE is also membrane-bound [8,12] and during platelet activation a particular portion of the enzyme is released [13]. Sheep platelet plasma membrane-bound AChE can be released by either *Staphylococcus aureus* phosphatidylinositol-specific phospholipase C (PI-PLC) or deoxycholate-specific platelet PI-PLC [14], although the exact nature of its isoforms is unknown.

In this study, we report the characteristics of AChE molecular forms extracted sequentially from sheep platelets with saline and saline-octylglucoside buffers. The dimeric molecular form obtained by sucrose gradient sedimentation analysis in membrane-bound AChE was confirmed after treatment with reducing and alkylating agents. The amphiphilic and hydrophilic properties of the enzyme were assessed by hydrophobic chromatography on phenyl-agarose and sedimentation analysis.

## 2. Materials and methods

### 2.1. Chemicals

Acetylthiocholine chloride, antiproteinases, bovine serum albumin (BSA), 1,5′-bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide (BW284c51), Brij 97, butyrylthiocholine chloride, 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), dithiothreitol (DTT), eserine (physostigmine), ethopropazine, *E. coli* β-D-galactosidase (16.0 $S_{20,w}$), bovine intestinal mucosa alkaline phosphatase (6.1 $S_{20,w}$), *N*-ethylmaleimide (NEM), β-D-octylglucoside (OG), Triton X-100 and phenyl-agarose were all from Sigma (Sigma-Aldrich Química, Spain). Ethylenediaminetetraacetic acid (EDTA), sucrose and the remaining products were provided by Panreac or Probus (Barcelona, Spain).

### 2.2. Solubilization of AChE

Platelets were isolated from the fresh blood of adult sheep (*Ovis aries* L. var. *domestica*) collected at a local slaughterhouse by the procedure previously described [8] using citrate-phosphate-dextrose-adenine as anticoagulant. Blood was spun at $300\times g$ for 15 min and the platelet-rich plasma was freed of other blood cells by three cycles of differential centrifugation at $1500\times g$ for 3 min, finally containing a low degree of contamination by erythrocytes ($<0.05\%$) and leukocytes ($<0.005\%$). The platelet preparation was sedimented at $3000\times g$ for 30 min, gently suspended in 10 mM Tris-HCl, pH 7.4 (buffer A), containing 0.15 M NaCl, and washed twice in the same buffered solution. Then, platelets resuspended at 50% (w/v) in buffer A containing 0.15 M NaCl and antiproteinases (1 mM EDTA and 1 mg/ml pepstatin, leupeptin and aprotinin) were homogenized with a Polytron as indicated previously [8]. A soluble extract (LSS fraction) was obtained after centrifuga-

tion of the homogenate at 105 000×*g* for 1 h at 4°C. The pellet was then extracted with a high-salt buffer (buffer A containing 1 M NaCl and 50 mM $MgCl_2$: buffer B) at 50% (w/v) by shaking for 30 min at 4°C. After centrifugation as above, the supernatant was recovered (HSS fraction). The pellet obtained, resuspended in buffer B, was treated with detergent (11 mg OG/mg protein or Triton X-100 under the previously reported conditions [15]) and centrifuged in the same conditions as indicated before, yielding a new supernatant fraction (DS fraction). Finally, non-extractable AChE was detected in the residual pellet ($P_3$ fraction). The distribution of AChE forms in the solubilized fractions was assessed by phenyl-agarose chromatography and centrifugation on sucrose gradients.

### 2.3. Separation of amphiphilic and hydrophilic forms of AChE

Amphiphilic and hydrophilic AChE molecules were separated by chromatography on a hydrophobic matrix and later identified by sedimentation analysis. Aliquots (5–10 ml) of the LSS, HSS and DS fractions containing 11 mg OG/mg protein were poured into a column (10×1 cm) of phenyl-agarose preequilibrated in buffer B. The column was then eluted (15 ml/h) with 75 ml of buffer B and subsequently with buffer B containing 2% (w/v) Triton X-100 (detergent-buffer B), collecting fractions of 2.5 ml and assaying for proteins and AChE activity. Hydrophilic forms were not retained by the gel, and retained amphiphilic AChE molecules were eluted with detergent-buffer B.

### 2.4. Sedimentation analysis

The molecular forms of AChE from the LSS, HSS and DS fractions and hydrophilic and amphiphilic peaks were analyzed by centrifugation on 5–40 or 5–20% (w/v) linear sucrose gradients made up in buffer B containing 0.5% Triton X-100 or 0.5% Brij 97 (w/v). Ultracentrifugation was performed at 165 000×*g* for 18 h. About 40 fractions (275 μl each) were collected from the bottom of each gradient and were assayed for AChE activity and enzyme markers. The relative proportions of the AChE activity of each molecular form were estimated by adding the activities under each peak and under the entire sedimentation profile (recovery between 90 and 95%). Sedimentation coefficients were calculated according to Martin and Ames [16], using β-galactosidase (16.0 $S_{20,w}$) and alkaline phosphatase (6.1 $S_{20,w}$) as standards. The hydrophilic or amphiphilic nature of the molecular forms was confirmed by their migration in sucrose gradients made up with Triton X-100 or Brij 97 [17].

### 2.5. Disulfide bridge reduction

In some experiments, samples from the amphiphilic AChE forms from DS extracts were treated with DTT at 10 mM final concentration at 37°C for 90 min at pH 8.6 in the presence or absence of 0.5 mM edrophonium chloride. The reappearance of disulfide bonds was prevented by addition to the mixture of NEM at 10 mM final concentration [18]. Reduced samples were dialyzed before sedimentation analysis to eliminate substances that might interfere with the assay of AChE activity.

### 2.6. Enzymatic assays

AChE and butyrylcholinesterase (BuChE) activities were measured by the method of Ellman et al. [19]. AChE was determined by measuring the hydrolysis of acetylthiocholine chloride as indicated previously [15]. BuChE activity was measured in a similar way, using 1 mM butyrylthiocholine as substrate in the presence of 10 μM BW284c51, a relatively specific inhibitor of AChE. One unit of AChE or BuChE activity is equal to 1 μmol of substrate hydrolyzed per min at 25°C. The AChE activity in fractions recovered from sucrose gradients was measured by application of a microtiter assay as described earlier [20]. Before substrate addition, samples were incubated for 15 min with DTNB and inhibitors to be sure that free thiol groups had reacted with the Ellman reagent and that BuChE had been completely inhibited by the appropriate inhibitor.

The protein contents of different samples were estimated by the adaptation of the Lowry method described by Dulley and Grieve [21], using BSA as standard.

## 3. Results and discussion

### 3.1. Solubilization of AChE

As has been reported previously [8,13], platelet AChE is distributed in both the cytosolic and membrane fractions. We were therefore testing the relative amounts of AChE activity of these two compartments in sheep platelets. It was found that after platelet homogenization with low-salt buffer containing antiproteinase mixture, about 30% of the enzyme activity and 78% of total protein could be recovered in cytosolic fraction (LSS) (Fig. 1). The remaining AChE activity was membrane-bound. These results are in fair agreement with those found previously by us [8]. The enzyme extracted in the cytosolic fraction represents the soluble AChE located or released during platelet activation in this fraction, as has been reported previously [13].

Owing to the observed heterogeneity in the solubilization behavior of several different AChE species, a sequential extraction procedure was applied. The results are given in Fig. 1. Treatment of sheep platelet crude membranes with high-salt buffer (buffer B) solubilized 14% of total proteins and a small amount of AChE (7% of total activity) in the HSS fraction, most of the activity remaining bound to the membrane. Similar results have been reported in human erythrocyte ghosts with buffers containing at least 1 M NaCl [11]. This finding suggests that the pool of solubilized AChE found in the HSS fraction might represent the loosely membrane-bound enzyme released during treatment with high ionic strength buffer. Subsequent treatment of the sheep platelet crude membranes, containing about 65% of total AChE, with high-salt buffer containing β-D-octylglucoside at 11 mg of detergent per mg of protein resulted in almost complete solubilization (bringing 92% of this enzyme activity into solution (DS fraction)) of the enzyme, which had a specific activity of 87.9 mU $mg^{-1}$, while 8% was not extracted (fraction $P_3$). Triton X-100 was less effective as a solubilizing agent because only 60% of the membrane-bound enzyme activity could be brought into solution at 1.5% (w/v) of detergent [15]. These results demonstrate that most of the enzyme is tightly bound to the membrane and are in agreement with those reported by Wong et al. [22], who demonstrated that, of the non-

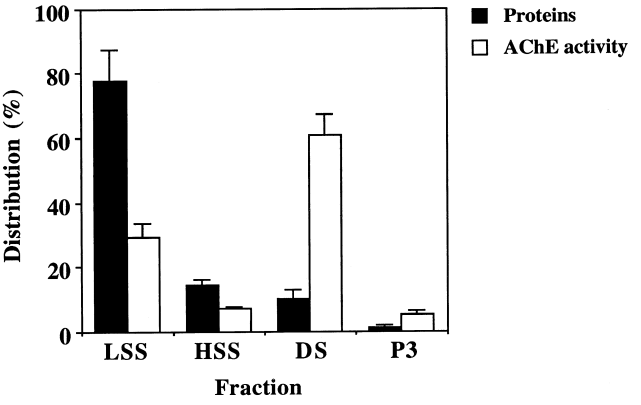


Fig. 1. Differential solubilization of sheep platelet AChE using saline buffers with or without OG detergent. Platelets were resuspended in low-salt buffer containing antiproteinases, homogenized and centrifuged at $105\,000 \times g$ for 1 h, recovering the cytosolic fraction (LSS). The crude membrane fraction was extracted with high-salt buffer followed by centrifugation as before, to yield a second soluble fraction (HSS). Finally, re-extraction of the pellet with high-salt buffer containing OG detergent followed by centrifugation at $105\,000 \times g$ for 1 h afforded a new supernatant fraction (DS) and a residual pellet ($P_3$). For specific details, see Section 2. The distribution of proteins and AChE activity in each fraction is expressed in percentages, referring the sum of the total amount of either proteins or AChE activity from the four fractions (LSS, HSS, DS and $P_3$) to 100%. Values are means ± S.E.M. of 4 individual determinations done in triplicate.

ionic detergents examined, the polyoxyethylene-based Tweens were the least effective detergents for the extraction of AChE from bovine erythrocyte membranes. This detergent-dependent differential solubilization pattern suggests that the AChE from membrane blood cells is linked to the membrane by a GPI anchor, as proposed by Hooper et al. [23]. In fact, in the absence of detergents, this enzyme can be partially solubilized from platelet membranes [14] and certain erythrocytes [4] by the action of PI-PLC. The glycolipid domain is responsible for detergent-enzyme interactions, affording the hydrophobic character [24].

### 3.2. Separation of amphiphilic and hydrophilic forms

The amphiphilic or hydrophilic properties of sheep platelet AChE were analyzed by hydrophobic chromatography on phenyl-agarose.

It is well known that hydrophilic forms are freely eluted in phenyl-agarose gel while amphiphilic forms

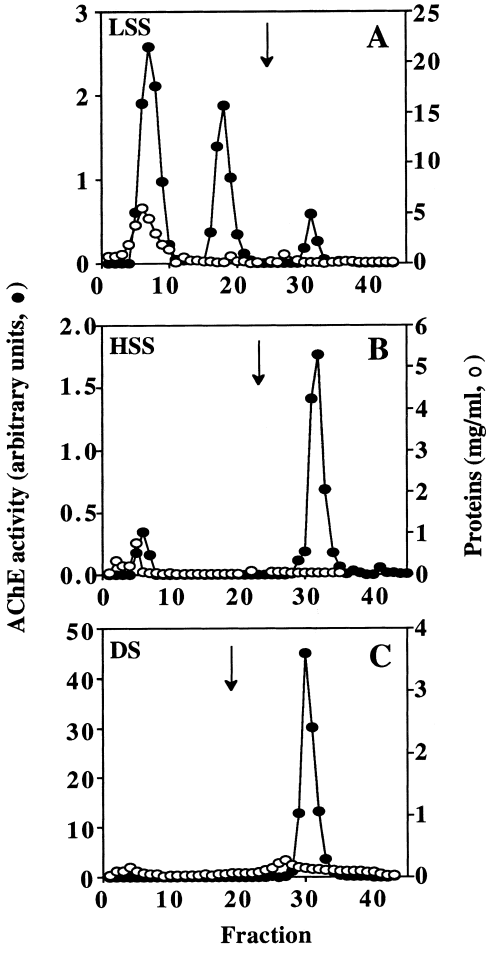


Fig. 2. Separation by phenyl-agarose chromatography of hydrophilic and amphiphilic AChE forms from LSS (panel A), HSS (panel B) and DS (panel C) fractions extracted from sheep platelets. The hydrophilic forms were eluted with the equilibrium buffer (buffer B) and the adsorbed amphiphilic forms with the equilibration buffer containing 2% Triton X-100 (w/v) (↓). The profiles shown are the results from a representative experiment of five individual experiments run.

remain bound to the gel. However, cholinesterases solubilized with Triton X-100 are unable to interact with the phenyl-agarose matrix, which prevents the separation of their amphiphilic and hydrophilic forms by this procedure [25]. Here we report for the first time that the replacement of Triton X-100 by OG in solubilization procedure permits the application of phenyl-agarose chromatography for this purpose, as described below. Because blood serum is rich in hydrophilic forms of BuChE [26], it was used in preliminary studies to gain insight into the behavior of these forms in the presence or absence of OG during phenyl-agarose chromatography. Samples from sheep serum with or without OG were applied on a phenyl-agarose column. As expected, the BuChE from samples without OG was not adsorbed onto the hydrophobic matrix. Similar results were found in samples containing OG, where about 90% of total activity was eluted with the equilibrium buffer (buffer B) and 9% with equilibrium buffer containing Triton X-100 (results not shown). Similar studies carried out in sheep platelet membrane-bound AChE, an amphiphilic protein as determined by Triton X-114 phase-partition [27], after solubilization with OG revealed that the enzyme was fully retained by the gel and was completely eluted after washing the column with Triton-equilibrium buffer (profiles identical to those shown in Fig. 2, panel C). However, when the enzyme was solubilized with Triton X-100, the enzyme extracted was not retained by the hydrophobic gel. The depletion of Triton X-100 by passage of the enzymatic extract through Bio-Beads SM-2 polystyrene resin and subsequent addition of OG allowed the gel-enzyme interaction to be recovered (data not shown). These results demonstrate that the addition of OG detergent does not modify the interaction of the enzyme with the hydrophobic gel, allowing the retention of amphiphilic forms and the free elution of hydrophilic forms in a similar manner to that reported for cholinesterases solubilized without detergent.

Study of the amphiphilic and hydrophilic properties of sheep platelet AChE was carried out in samples extracted with low-salt buffer (fraction LSS), high-salt buffer (fraction HSS) and OG detergent-high salt buffer (fraction DS). The fractions containing OG-detergent were applied on phenyl-agarose columns and afforded the elution profiles shown in Fig. 2. In samples from the LSS fraction, most of the AChE (90%) was freely eluted in the chromatographic process and the remaining enzyme (7%) bound to the gel was recovered by passing Triton-equilibrium buffer through the column. A typical separation profile is shown in panel A of Fig. 2. The hydrophilic forms, which represent about 90% of the total AChE in this fraction, are separated into two peaks (I and II) with variable proportions; in this case, 57 and 35%. Soluble AChE (fraction LSS) contained the highest percentage of hydrophilic forms, with 27% of total AChE extracted, whereas

only trace amounts (about 3%) of amphiphilic forms were detected (Table 1). This distribution is similar to that found for naturally soluble cholinesterases from other materials, where a predominance of hydrophilic forms and a small proportion of amphiphilic forms have also been described [1,3,25,28,29]. The hydrophobic character of amphiphilic forms found in soluble AChE is probably masked by other amphiphilic molecules (lipids or other amphiphiles) to form a soluble complex that is not pelleted. It should be noted in this respect that detergent-interacting forms of AChE also occur in the low-salt soluble fraction of electric organs [30] and in aqueous extracts of housefly heads [31]. The amphiphilic and a significant portion of hydrophilic forms found in sheep platelet soluble AChE (LSS fraction) could come from membrane-bound amphiphilic forms after platelet activation or during enzyme solubilization. Hydrophilic forms would merely be products generated by the action of endogenous proteases or phospholipases, as suggested in *Musca* and *Drosophila* by autolysis phenomena [29,31,32]. In fact, the AChE of sheep platelet membrane is sensitive to PI-PLC [14].

After phenyl-agarose chromatography, $12.3 \pm 0.6\%$ ($n = 5$) of the sheep platelet AChE loosely bound to the membrane and extracted with high-salt buffer in the HSS fraction was eluted by passing the equilibrium buffer through the column while the remaining activity ($84.9 \pm 1.2$, $n = 5$) was eluted after washing with Triton-equilibrium buffer (Fig. 2, panel B). From these data, the percentage distribution of amphiphilic and hydrophilic forms of AChE from this fraction was calculated (Table 1); it was found that 6.1% of total AChE were amphiphilic forms and only 0.9% had a hydrophilic character. The high proportion of amphiphilic forms found in this fraction suggests a membrane origin, and these forms may be released during enzyme extraction with high ionic strength buffer, as has been established in erythrocyte ghosts by treatments with buffers containing at least 1 M NaCl [11]. Our data contrast with those reported for mammalian brain, where most of the AChE released during homogenization with high salt buffers displays hydrophilic characteristics and only 25% are amphiphilic forms [25]. These data are in accordance with the general idea that in many tissues extraction with low or high salt buffers releases a certain amount of AChE containing completely water-soluble forms and other forms that interact with non-denaturing detergents, although detachment of the bulk of the membrane-bound enzyme requires incubation of the tissue with detergents [3,11].

The tightly membrane-bound AChE in sheep platelets extracted with detergent-high salt buffer in the DS fraction was retained by phenyl-agarose gel and eluted with Triton-equilibrium buffer in a single activity peak ($97.2 \pm 0.5\%$ of total activity in this fraction, $n = 5$) (Fig. 2, panel C). This activity corresponds to amphiphilic forms, which represent about 59% of total AChE in sheep platelets (Table 1). Hydrophilic forms are absent in this fraction. By contrast, in other sources detergent-soluble fractions often contain varying proportions of hydrophilic forms occluded within intracellular vesicles that require detergents only for solubilization [33,34]. The exclusive amphiphilic nature reported in AChE solubilized in the DS fraction is in agreement with the notion that membrane-bound AChE from sheep platelets is recovered mainly (85%) in the detergent-rich phase during phase separation with Triton X-114 [27]. This behavior is also in accordance with the hydrophobic glycolipid anchor postulated for sheep platelet membrane-bound AChE, as suggested by the

Table 1
Percentage distribution of amphiphilic and hydrophilic AChE forms in solubilized fractions from sheep platelets

| Form | Fraction | | | Total percentage |
|---|---|---|---|---|
| | LSS | HSS | DS | |
| Hydrophilic | $26.9 \pm 3.1$ | $0.88 \pm 0.08$ | ND | $27.8 \pm 3.1$ |
| Amphiphilic | $2.81 \pm 0.6$ | $6.1 \pm 0.5$ | $59.3 \pm 0.7$ | $68.2 \pm 0.9$ |

Data were calculated from the expression $a \times b/100$, where 'a' represents the % of AChE in each solubilized fraction (each data point corresponds to each individual determination of the 4 used to make Fig. 1), and 'b' is the relative proportion of hydrophilic or amphiphilic forms in each fraction (data shown in the text of Section 3). ND: not detected.

treatment with PI-PLC [14]. A predominance of amphiphilic AChE forms in membranes from erythrocytes and lymphocytes from different sources [4,6], in the *Torpedo* electric organ [30,35] and in insect tissues [29], has also been found, the enzyme tending to form aggregates when the solubilizing detergent is removed. In some cases, these hydrophobic interactions may be suppressed by mild treatment with proteases or phospholipases [4,29,31], releasing a short hydrophobic domain at the C-terminus of each catalytic subunit and hydrophilic activity.

### 3.3. Molecular forms of AChE

The hydrodynamic characteristics of AChE forms extracted in the solubilized LSS, HSS and DS fractions and in amphiphilic and hydrophilic forms separated by phenyl-agarose chromatography were investigated by sedimentation analysis.

Preliminary experiments revealed that analysis of whole AChE in sucrose gradients with 0.15 M NaCl containing Triton X-100 or Brij 97 afforded broad enzyme profiles, enzyme activity being visible along the whole gradient (data not shown). These results probably indicate aggregation states of AChE. We therefore included high ionic strength buffers in the gradients to study the native molecular forms of the enzyme.

As can be seen in Fig. 3, sedimentation analysis of soluble AChE (LSS extract) in gradients revealed the occurrence of two main peaks. The major peak shifted from 5.6 S in Triton X-100 to 5.1 S in Brij 97, whereas the minor peak changed from 3 S in Triton X-100 to 2.1 S (shoulder) in Brij 97. The slight modifications detected by changes in the detergent during migration in gradients are probably a consequence of the very low contribution of amphiphilic forms present in this extract (about 3% of total AChE activity), as has been reported previously using phenyl-agarose chromatography. In agreement with previous data [36,37], the above AChE forms should correspond to globular dimers ($G_2$) and monomers ($G_1$), which represent about 80% and 20% of the total AChE in the fraction. Taking into account that the hydrophilic fraction corresponds to about 27% of total activity in soluble AChE, the above distribution suggests that dimeric hydrophilic forms would represent about 22% of total activity

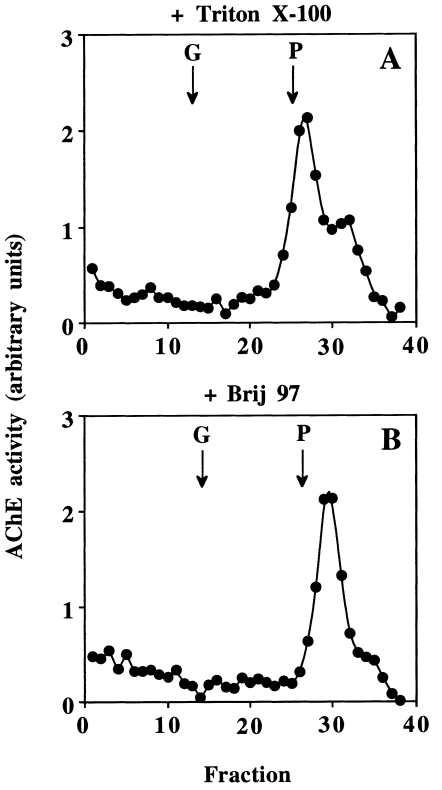


Fig. 3. Sedimentation profiles of cytosolic AChE from sheep platelets. The cytosolic fraction (LSS fraction) was analyzed by centrifugation on a linear sucrose gradient (5–20%) carried out in high-saline buffer (buffer B) with 0.5% Triton X-100 or 0.5% Brij 97 (w/v). Arrows (↓) show the position of enzyme markers in the gradient: β-galactosidase (16.0 S, G) and alkaline phosphatase (6.1 S, P). Note that the sedimentation coefficient values of the main peak (5.6 S, $G_2$ form) and shoulder (3 S, $G_1$ form) in the gradient made up with Triton X-100 were reduced in comparison with those obtained with Brij 97 (5.1 S and 2.1 S, respectively).

($G_2^H$) in the soluble enzyme, whereas the remainder would correspond to monomeric hydrophilic forms (5%, $G_2^A$). Sedimentation analysis of soluble AChE not retained by phenyl-agarose in gradients with Triton X-100 disclosed profiles with a main peak of 5.1–5.3 S, with no shift in the sedimentation coefficient when Triton X-100 was replaced by Brij 97 (data not shown). This kind of behavior corresponds to globular detergent-insensitive forms, probably dimers ($G_2^H$) and corroborates the above considerations. An amphiphilic dimer can be converted into its hydrophilic counterpart after different proteolytic [31,38,39] or bacterial PI-PLC [6,18,40] treatments or by autolysis [32]. Thus, the action of either sig-

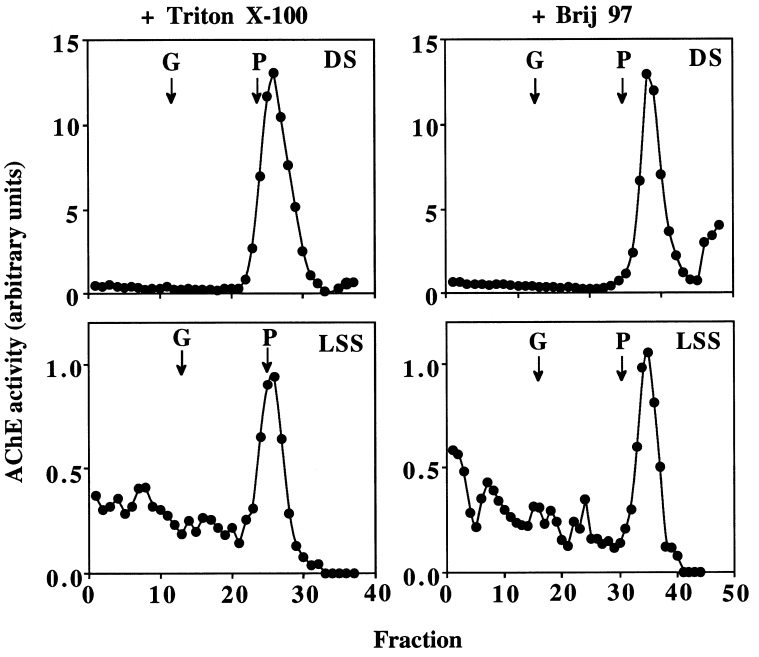


Fig. 4. Sedimentation profiles of amphiphilic AChE forms solubilized in DS (upper panels) and LSS (lower panels) fractions from sheep platelets. Amphiphilic forms separated by phenyl-agarose chromatography as indicated in Fig. 2 were layered onto a 5–20% (w/v) continuous sucrose gradient made up in high-saline buffer with 0.5% Triton X-100 or 0.5% Brij 97 and centrifuged at 165 000×*g* for 18 h. Internal markers as in Fig. 3. The sedimentation coefficients in gradients with Triton X-100 (5.2 S in DS and 5.6 S in LSS) shifted in the presence of Brij 97 (4.6 S in DS and 4.2 S in LSS).

nal-generating enzymes, such as PI-PLC, or that of endogenous proteases released during adhesion or platelet activation [41] could at least partially be responsible for the predominant hydrophilic dimeric forms of AChE found in sheep platelet LSS extracts. These molecular forms may represent a degradation product of the membrane-bound AChE form due to the progressive conversion of $G_2^A$ into $G_2^H$ and $G_1^H$ as a result of the loss of the glycolipid domain, as has been postulated in rabbit lymphocytes [6] and observed in fresh extracts of housefly heads even in the presence of high concentrations of antiproteinases [32]. The monomers found in LSS extracts from sheep platelets could arise through the action of either endogenous proteases or residual endogenous reducing agents [42]. It is well known that reduction by dithiothreitol or mercaptoethanol converts amphiphilic and hydrophilic dimers into amphiphilic and hydrophilic monomers, respectively [5,11,24,31,42]. However, the inclusion of NEM during enzyme extraction prevents the conversion of $G_2$ to $G_1$, apparently through the alkylation of sulfhydryl groups of endogenous reducing agents or proteases, as has been found in chicken AChE [43] and human erythroleukemia K562 cells [18]. Minor amounts of monomeric enzyme have also been observed in different sources, such as human erythrocyte membranes [11], two sublines of human erythroleukemia K562 cells [18], human lymphocyte membranes [5], insect extracts [29] and several vertebrate tissues [3].

Sedimentation analysis of amphiphilic AChE separated by phenyl-agarose chromatography from LSS and DS extracts revealed a main peak sedimenting at 5.6 S and 5.2 S, respectively, in the gradients containing Triton X-100, as can be seen in Fig. 4. These sedimentation coefficients were slightly shifted to 4.2 S and 4.6 S, respectively, in the presence of Brij 97, confirming that they were indeed amphiphilic forms. These profiles observed in 5–20% sucrose gradients were identical to those found when 5–40% sucrose gradients were used. Similar sedimentation profiles were found in amphiphilic AChE extracted in the HSS fraction, where sedimentation coefficients of 5.2 S and 4.5 S were detected in the presence of Triton X-100 and Brij 97, respectively (data not

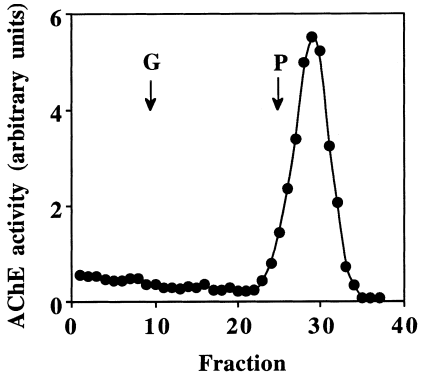


Fig. 5. Sedimentation analysis of amphiphilic forms of membrane-bound AChE from sheep platelets after reduction and alkylation. Samples obtained as indicated in Fig. 2 were reduced with 10 mM DTT at 37°C for 90 min at pH 8.6 in the presence of 0.5 mM edrophonium chloride, adding NEM at a 10 mM final concentration. Then, dialyzed samples were loaded onto a 5–20% (w/v) linear sucrose gradient made up in high-saline buffer containing 0.5% Triton X-100 and centrifuged at $165\,000 \times g$ for 18 h. Internal markers as in Fig. 3. The sedimentation value of the single peak (form $G_1$) detected in the gradients was 4 S.

shown). The sedimentation profiles obtained in unfractionated AChE from the HSS and DS fractions were almost identical to those shown in Fig. 4, with a main peak sedimenting at about 6 S in gradients with Triton X-100 (data not shown). These mean S values ($5.6 \pm 0.7$ S in Triton X-100 and $4.6 \pm 0.3$ S in Brij 97, $n = 4$) are similar to those previously determined for $G_2$ AChE from human erythroleukemia K562 cells [18], rabbit and human erythrocytes [6,11,24], several insects [29] and *Torpedo* electric organ [30], and probably correspond to the dimeric state of the globular catalytic subunit of the enzyme. These assignations were confirmed following the reduction of disulfide bonds with dithiothreitol, a treatment which converts dimers to monomers without altering detergent interactions [18,24]. The reduction procedure in the presence of the sulfhydryl alkylating agent NEM resulted in some loss of enzyme activity, but this loss was reduced almost two-fold by the addition of 0.5 M edrophonium chloride, a competitive inhibitor of AChE, to the reduction medium. Density gradient analysis in the presence of Triton X-100 of DS extracts after reduction and alkylation pointed to a single activity peak of AChE with an apparent S value of 4 S, regardless of the presence or absence of edrophonium in the reduction medium (Fig. 5). This sedimentation coefficient is consistent with a globular monomeric form [1,5,11,18,24]. In any case, the fact that this coefficient value is lower than that found in AChE from non-reduced DS extracts clearly indicates that the amphiphilic AChE molecular forms detected by us are $G_2$ rather than $G_1$ forms. These dimeric molecular forms must correspond to native forms anchored in sheep platelet membrane by a glycolipid domain, as suggested by Majumdar and Balasubramanian [14]. This molecular form is probably similar to the globular dimeric forms described in Diptera AChE [29] and AChE from different vertebrate tissues, such as *Torpedo* electric organ [44], mammalian erythrocytes [4] and lymphocytes [6].

In summary, our results show that amphiphilic globular dimers are the dominant molecular form found in sheep platelet AChE, and can be mostly extracted mainly by treatment with detergent-buffer or, in minor amounts, after treatment with low and high ionic strength buffers without detergents. We suggest that the predominance of hydrophilic forms found in soluble AChE (LSS extracts) could be the result of a significant conversion of $G_2^A$ into $G_2^H$ and $G_1^H$ through the action of either endogenous proteases and phospholipases or residual endogenous reducing agents.

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## References

[1] J. Massoulié, L. Pezzementi, S. Bon, E. Krejci, F.M. Vallette, Molecular and cellular biology of cholinesterases, Prog. Neurobiol. 41 (1993) 31–91.
[2] P. Taylor, Z. Radic, The cholinesterases; from genes to proteins, Annu. Rev. Pharmacol. Toxicol. 34 (1994) 281–320.
[3] J. Massoulié, S. Bon, The molecular forms of cholinesterase and acetylcholinesterase in vertebrates, Annu. Rev. Neurosci. 5 (1982) 57–106.
[4] I. Silman, A.H. Futerman, Modes of attachment of acetylcholinesterase to the surface membrane, Eur. J. Biochem. 170 (1987) 11–22.

[5] E. Bartha, Z. Rakonczay, P. Kása, S. Hollán, A. Gyévai, Molecular form of human lymphocyte membrane-bound acetylcholinesterase, Life Sci. 41 (1987) 1853–1860.
[6] P. Richier, M. Arpagaus, J.P. Toutant, Glycolipid-anchored acetylcholinesterases from rabbit lymphocytes and erythrocytes differ in their sensitivity to phosphatidylinositol-specific phospholipase C, Biochim. Biophys. Acta 1112 (1992) 83–88.
[7] S.D. Shukla, Action of phosphatidylinositol specific phospholipase C on platelets nonlytic release of acetylcholinesterase, effect of thrombin and PAF induced aggregation, Life Sci. 38 (1986) 751–755.
[8] J. Sánchez-Yagüe, J.A. Cabezas, M. Llanillo, Subcellular distribution and characterization of acetylcholinesterase activities from sheep platelets: relationship between temperature-dependence and environment, Blood 76 (1990) 737–744.
[9] A.S. Balasubramanian, C.D. Bhanumathy, Noncholinergic functions of cholinesterases, FASEB J. 7 (1993) 1354–1358.
[10] J. Massoulié, J.-P. Toutant, Vertebrate cholinesterases: structure and types of inhibition, Handbook Exp. Pharmacol. 86 (1988) 167–224.
[11] P. Ott, Membrane acetylcholinesterase: purification, molecular properties and interactions with amphiphilic environments, Biochim. Biophys. Acta 822 (1985) 375–392.
[12] R. Majumdar, S.T. George, A.S. Balasubramanian, Serotonin-sensitive arylacylamidase activity of platelet acetylcholinesterase, Biochem. Pharmacol. 31 (1982) 2319–2325.
[13] H.Y.K. Chuang, S.F. Mohammad, R.G. Mason, Acetylcholinesterase, choline acetyltransferase and the postulated acetylcholine receptor of canine platelets, Biochem. Pharmacol. 25 (1976) 1971–1977.
[14] R. Majumdar, A.S. Balasubramanian, The solubilization of platelet membrane-bound acetylcholinesterase and arylacylamidase by exogenous or endogenous phosphatidylinositol specific phospholipase C, Biochem. Pharmacol. 34 (1985) 4109–4115.
[15] E.M. Martín-Valmaseda, J. Sánchez-Yagüe, J.A. Cabezas, M. Llanillo, Biochemical characterization of sheep platelet acetylcholinesterase after detergent solubilization, Comp. Biochem. Physiol. 110B (1995) 91–101.
[16] R.G. Martin, B.N. Ames, A method for determining the sedimentation behaviour of enzymes. Applications to protein mixtures, J. Biol. Chem. 236 (1961) 1372–1379.
[17] J. Massoulié, J.P. Toutant, in: V.P. Whittaker (Ed.), Handbook of Experimental Pharmacology, Vol. 86, Springer, Heidelberg, 1988, pp. 167–224.
[18] J.P. Toutant, M.K. Richards, J.A. Krall, T.L. Rosenberry, Molecular forms of acetylcholinesterase in two sublines of human erythroleukemia K562 cells, Eur. J. Biochem. 187 (1990) 31–38.
[19] G.L. Ellman, K.D. Courtney, V. Andres, R.M. Featherstone, A new and rapid colorimetric determination of acetylcholinesterase activity, Biochem. Pharmacol. 7 (1961) 88–95.
[20] M.R. Moya-Quiles, J. Villalba-Sánchez, E. Muñoz-Delgado, C.J. Vidal, Alkaline treatment of muscle microsomes releases amphiphilic and hydrophilic forms of acetylcholinesterase, Biochim. Biophys. Acta 1121 (1992) 88–96.
[21] J.R. Dulley, P.A. Grieve, A simple technique for eliminating interference by detergents in the Lowry method of protein determination, Anal. Biochem. 64 (1975) 136–141.
[22] R.K.M. Wong, C.P. Nichol, M.C. Sekar, B.D. Roufogalis, The efficiency of various detergents for extraction and stabilization of acetylcholinesterase from bovine erythrocytes, Biochem. Cell Biol. 65 (1987) 8–18.
[23] N.M. Hooper, in: N.M. Hooper, A.J. Turner (Eds.), Lipid Modification of Proteins: A Practical Approach, IRL Press, Oxford, 1992, pp. 89–115.
[24] T.L. Rosenberry, D.M. Scoggin, Structure of human erythrocyte acetylcholinesterase. Characterization of intersubunit disulfide bonding and detergent interaction, J. Biol. Chem. 259 (1984) 5643–5652.
[25] J. Saéz-Valero, P.L. Tornel, E. Muñoz-Delgado, C.J. Vidal, Amphiphilic and hydrophilic forms of acetyl- and butyrylcholinesterase in human brain, J. Neurosci. Res. 35 (1993) 678–689.
[26] O. Lockridge, Structure of human serum cholinesterase, BioEssays 9 (1988) 125–128.
[27] A. Hernández-Hernández, J. Sánchez-Yagüe, E.M. Martín-Valmaseda, M. Llanillo, Characterization of phosphotyrosine phosphatase activity in sheep platelets. Amphiphilic and hydrophilic forms, Comp. Biochem. Physiol. 117B (1997) 437–444.
[28] V. Talesa, M. Grauso, E. Giovannini, G. Rosi, J.P. Toutant, Solubilization, molecular forms, purification and substrate specificity of two acetylcholinesterases in the medicinal leech (*Hirudo medicinalis*), Biochem. J. 306 (1995) 687–692.
[29] J.P. Toutant, Insect acetylcholinesterase: catalytic properties, tissue distribution and molecular forms, Prog. Neurobiol. 32 (1989) 423–446.
[30] S. Bon, J. Massoulié, Collagen-tailed and hydrophobic components of acetylcholinesterase in *Torpedo marmorata* electric organ, Proc. Natl. Acad. Sci. USA 77 (1980) 4464–4468.
[31] D. Fournier, A. Cuany, J.M. Bride, J.B. Bergé, Molecular polymorphism of head acetylcholinesterase from adult houseflies, J. Neurochem. 49 (1987) 1455–1461.
[32] J.P. Toutant, M. Arpagaus, D. Fournier, Native molecular forms of head acetylcholinesterase from adult *Drosophila melanogaster*: Quaternary structure and hydrophobic character, J. Neurochem. 50 (1988) 209–218.
[33] S. Bon, Molecular forms of acetylcholinesterase in developing *Torpedo* embryos, Neurochem. Int. 4 (1982) 577–585.
[34] M. Arpagaus, J.P. Toutant, Polymorphism of acetylcholinesterase in adult *Pieris brassicae* heads. Evidence for detergent-insensitive and Triton X-100-interacting forms, Neurochem. Int. 7 (1985) 793–804.
[35] A.H. Futerman, M.G. Low, I. Silman, A hydrophobic dimer of acetylcholinesterase from *Torpedo californica* electric organ is solubilized by phosphatidylinositol-specific phospholipase C, Neurosci. Lett. 40 (1983) 85–89.
[36] J.P. Toutant, J. Massoulié, in: A.J. Kenny, A.J. Turner,

(Eds.), Mammalian Ectoenzymes, Elsevier, Amsterdam, 1987, pp. 289–328.
[37] M.T. Moral-Naranjo, J. Cabezas-Herrera, C.J. Vidal, Molecular forms of acetylcholinesterase and butyrylcholinesterase in normal and dystrophic mouse brain, J. Neurosci. Res. 43 (1996) 224–234.
[38] T.A. Dutta-Choudhury, T.L. Rosenberry, Human erythrocyte acetylcholinesterase is an amphipathic protein whose short membrane-binding domain is removed by papain digestion, J. Biol. Chem. 259 (1984) 5653–5660.
[39] W.L. Roberts, T. Rosenberry, Selective radiolabeling and isolation of the hydrophobic membrane-binding domain of human erythrocyte acetylcholinesterase, Biochemistry 25 (1986) 3091–3098.
[40] M.G. Low, J.B. Finean, Nonlytic release of acetylcholinesterase by a phosphatidylinositol-specific phospholipase C, FEBS Lett. 82 (1977) 143–146.
[41] A.J. Marcus, L.B. Safier, Thromboregulation: multicellular modulation of platelet reactivity in hemostasis and thrombosis, FASEB J. 7 (1993) 516–522.
[42] A.L. Gnagey, M. Forte, T.L. Rosenberry, Isolation and characterization of acetylcholinesterase from *Drosophila*, J. Biol. Chem. 262 (1987) 13290–13298.
[43] R.L. Rotundo, D.M. Fambrough, Molecular forms of chicken embryo acetylcholinesterase in vitro and in vivo, isolation and characterization, J. Biol. Chem. 254 (1979) 4790–4799.
[44] S. Bon, J.P. Toutant, K. Méflah, J. Massoulié, Amphiphilic and non amphiphilic forms of *Torpedo* acetylcholinesterase. II. Existence of electrophoretic variants and of phosphatidylinositol phospholipase C-sensitive and -insensitive forms, J. Neurochem. 51 (1988) 786–794.